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van den Heuvel, RHH; Fraaije, MW; Laane, C; van Berkel, WJH; Heuvel, Robert H.H. van den; Berkel, Willem J.H. van

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Enzymatic Synthesis of Vanillin

Robert H. H. van den Heuvel,[†] Marco W. Fraaije,[‡] Colja Laane,[†,§] and Willem J. H. van Berkel*,[†]

Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands, and Laboratory of Biochemistry, Department of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Due to increasing interest in natural vanillin, two enzymatic routes for the synthesis of vanillin were developed. The flavoprotein vanillyl alcohol oxidase (VAO) acts on a wide range of phenolic compounds and converts both creosol and vanillylamine to vanillin with high yield. The VAO-mediated conversion of creosol proceeds via a two-step process in which the initially formed vanillyl alcohol is further oxidized to vanillin. Catalysis is limited by the formation of an abortive complex between enzyme-bound flavin and creosol. Moreover, in the second step of the process, the conversion of vanillyl alcohol is inhibited by the competitive binding of creosol. The VAO-catalyzed conversion of vanillylamine proceeds efficiently at alkaline pH values. Vanillylamine is initially converted to a vanillylimine intermediate product, which is hydrolyzed nonenzymatically to vanillin. This route to vanillin has biotechnological potential as the widely available principle of red pepper, capsaicin, can be hydrolyzed enzymatically to vanillylamine.

Keywords: *Capsaicin; creosol; flavoprotein; vanillin; vanillyl alcohol oxidase; vanillylamine*

INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a widely used flavor compound in food and personal products (*1*), and in high concentrations, the antioxidizing properties of this compound prevent oxidative damage in mammalian cells (*2*, *3*). By far the dominant route for vanillin production is chemical synthesis from lignin, coniferin, the glucoside of coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol), guaiacol (2-methoxyphenol), or eugenol (4-allyl-2-methoxyphenol) (*1*). Natural vanillin supplies <1% of the total demand for vanillin and is produced from glucovanillin when the beans of the orchid *Vanilla planifolia* are submitted to a multistep curing process. After this curing process, vanillin is the most abundant component of the bean at a level of ∼2−3 wt % (*4*, *5*).

With the increasing interest in natural products, alternative processes are being developed to produce natural vanillin. The term "natural" can be applied both in the European Union and in the United States when the product has been derived from a natural raw material via biological (e.g., enzymes or whole cells) and/or mild processing tools (e.g., extraction or distillation) (*6*). In recent years, a large number of studies have been made on natural vanillin biosynthesis using microorganisms or isolated enzymes (*7*−*12*). However, these bioconversions are not yet economically feasible.

The flavoprotein vanillyl alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* (*13*) is a versatile biocatalyst that is of particular interest for the production of vanillin. Eugenol (4-allyl-2-methoxyphenol) is the principal constituent of clove oil and is an economically realistic feedstock. VAO catalyzes the hydroxylation of eugenol to coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) (*14*, *15*). This aromatic alcohol can be further converted, via ferulic acid (4-hydroxy-3-methoxycinnamic acid), to vanillin by several microorganisms (*1*, *12*).

VAO can also produce vanillin by catalyzing the oxidation of vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), the oxidative demethylation of 2-methoxy-4-(methoxymethyl)phenol, and the oxidative deamination of vanillylamine (4-hydroxy-3-methoxybenzylamine) (*14*) (Figure 1A−C). Vanillyl alcohol, 2-methoxy-4-(methoxymethyl)phenol, and vanillylamine are not widely available in nature and thus not of direct interest for biotechnological applications. However, vanillylamine can be obtained from the abundant precursor compound capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent principle of hot red pepper, by the cleavage of its amide bond (*16*, *17*) (Figure 1D). Another potential feedstock for vanillin is creosol (2-methoxy-*p*-cresol), which is the major component in creosote obtained from heating wood or coal tar (*18*, *19*). On the basis of the reactivity of VAO with *p*-cresol it was anticipated that creosol is oxidized to vanillin (*20*) (Figure 1E).

In this study, we have investigated the VAO-mediated synthesis of vanillin from natural feedstocks. We selected creosol, which is the major product in creosote, and vanillylamine, as this compound can be obtained from capsaicin.

MATERIALS AND METHODS

Materials. Creosol (2-methoxy-*p*-cresol), vanillylamine (4-hydroxy-3-methoxybenzylamine), vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), and vanillin (4-hydroxy-3-methoxybenzaldehyde) were obtained from Aldrich (Steinheim, Germany), and capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) was from Fluka (Buchs, Switzerland). Penicillin G acylase from *Escherichia coli* was a kind gift from DSM/Gist (Delft, The

* Author whom correspondence should be addressed (telephone +31-317-482861; fax +31-317-484801; e-mail willem.vanberkel@fad.bc.wau.nl).

[†] Wageningen University.

[‡] University of Groningen.

[§] Present address: DSM Food Specialties Research and Development, P.O. Box 1, 2600 MA Delft, The Netherlands.

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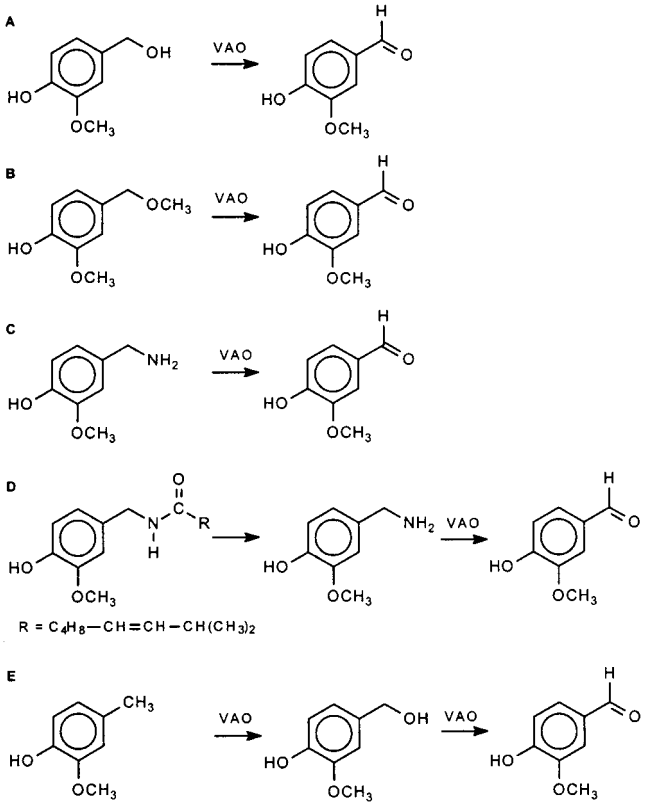

Figure 1. VAO-catalyzed conversion of (A) vanillyl alcohol, (B) 2-methoxy-4-(methoxymethyl)phenol, and (C) vanillylamine to vanillin and two-step enzymatic conversion of (D) capsaicin and (E) creosol to natural vanillin.

Netherlands). Lipase B from *Candida antarctica* was obtained from Novo Nordisk (Bagsværd, Denmark). Carboxypeptidase A from bovine pancreas, carboxypeptidase Y from yeast, acylase from pig kidney, and thermolysin from *Bacillus thermoproteolyticus* were purchased from Sigma (St. Louis, MO). All other chemicals were obtained as previously described (*21*, *22*). Rat liver microsomes were prepared essentially as described by Rietjens and Vervoort (*23*).

Expression and Purification of VAO. *E. coli* strain TG2 (*24*) and the plasmid pEMBL19 (Boehringer Mannheim) were used for expression of the *vao*A gene (*25*). Transformed *E. coli* cells were grown in Luria−Bertani medium supplemented with 75 μg/mL ampicillin and 0.25 mM isopropyl β-D-thiogalactopyranoside (*25*). VAO was purified as described to a purity of >98% (*22*, *25*).

Analytical Methods. VAO activity was determined at 25 °C by monitoring changes in the absorption spectra of aromatic products or by oxygen consumption experiments using a Clark electrode (*14*). Vanillin production was measured at 340 nm (ϵ_{340} varies from 2.2 mM/cm at pH 5.6 to ϵ_{340} = 23.3 mM/cm at pH 10.5 with a pK_a of 7.2) and vanillylimine production at 390 nm. For enzyme-monitored-turnover experiments, air-saturated enzyme and substrate solutions were mixed, and the redox state of the flavin prosthetic group was monitored at 439 nm using a Hewlett-Packard HP 8453 diode array spectrophotometer. Stopped-flow kinetics were performed with a Hi-Tech SF-51 apparatus equipped with a Hi-Tech SU-40 spectrophotometer, essentially as described previously (*26*). In anaerobic reduction experiments, glucose-containing enzyme solutions were flushed with oxygen-free argon gas and glucose oxidase was added to eliminate final traces of oxygen. Fluorescence emission spectra were recorded at 25 °C on an Aminco SPF-500C spectrofluorometer; the excitation wavelength was 360 nm (*20*).

HPLC experiments were performed with an Applied Biosystems pump equipped with a Waters 996 photodiode array detector and a 4.6 × 150 mm Alltima C18 column (Alltech) using mixtures of methanol and water containing 1% (v/v) acetic acid. For monitoring the conversion of creosol to vanillin a methanol/water ratio of 35:65 (v/v) was used. For monitoring the conversion of capsaicin to vanillylamine, elution was started with a linear gradient from 8 to 13% (v/v) methanol in 20 min followed by an increase to 75% in 3 min, which was held for 20 min. The VAO-mediated conversion of creosol was carried out in different buffers at 25 °C. The hydrolysis of capsaicin by penicillin G acylase was performed in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C, and the hydrolysis of capsaicin by rat liver microsomes was performed in 50 mM potassium phosphate buffer, pH 7.0, at 37 °C. Chemical hydrolysis of capsaicin was carried out in 10 or 100 mM NaOH at 95 °C. In all reaction mixtures capsaicin was added as a powder. The reactions were terminated by the addition of 5% (w/v) trichloroacetic acid and subsequent centrifugation.

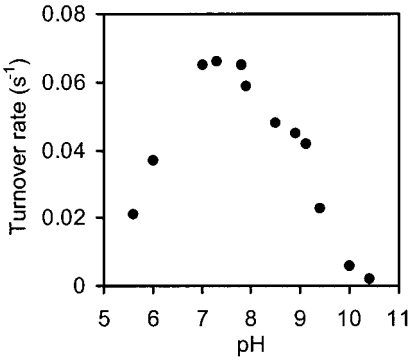

Figure 2. pH dependence of the reaction of VAO with creosol. The activity measurements were performed in 50 mM succinate/NaOH (pH 5.6−6.9), 50 mM potassium phosphate buffer (pH 6.1−7.8), 50 mM Tris/H_2SO_4 (pH 7.9−8.9), and 50 mM glycine/NaOH (pH 9.1−10.4) at 25 °C using 150 μM creosol and 0.5 μM VAO.

Table 1. Steady-State Kinetic Parameters for VAO in Air-Saturated 50 mM Potassium Phosphate Buffer, pH 7.5, at 25 °C

substrate	k'_{cat} (s^{-1})	K'_m (μM)	k'_{cat}/K'_m (s^{-1} mM^{-1})
4-(methoxymethyl)phenol[a]	3.1	55	56.4
vanillylamine	0.02	48	0.42
creosol	0.07	50	1.4
vanillyl alcohol[a]	3.3	160	20.6

[a] Data from ref *26*.

RESULTS

Conversion of Creosol. Table 1 summarizes the steady-state kinetic parameters of VAO with 4-(methoxymethyl)phenol, vanillyl alcohol, vanillylamine, and creosol at pH 7.5. 4-(Methoxymethyl)phenol and vanillyl alcohol are efficient substrates for VAO, whereas both vanillylamine and creosol react rather slowly with the enzyme. In contrast to the other substrates, the conversion of creosol proceeds via a two-step enzymatic process. In the first step creosol is hydroxylated by VAO to yield vanillyl alcohol, and in the second step vanillyl alcohol is oxidized to yield vanillin.

Earlier studies have shown that the reaction of VAO with 4-(methoxymethyl)phenol and vanillyl alcohol is optimal around pH 10 (*13*, *14*). However, when the VAO-mediated conversion of creosol was studied at pH 10, almost no activity was observed. Upon determining the pH optimum of the reaction, we found that VAO is most active with creosol between pH 7 and 8 (Figure 2). This pH optimum is identical to the optimum of the VAO-mediated oxidation of *p*-cresol (*20*) and clearly distinct

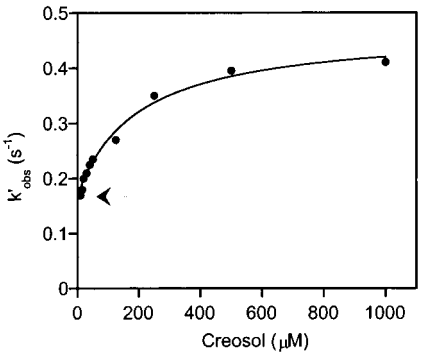

Figure 3. Observed reduction rates of VAO with various concentrations of creosol. VAO (2.5 μM) was mixed anaerobically with creosol (25−500 μM) in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. Flavin reduction was followed at 439 nm. The arrow indicates the value found for the reverse reaction (k_{-2}).

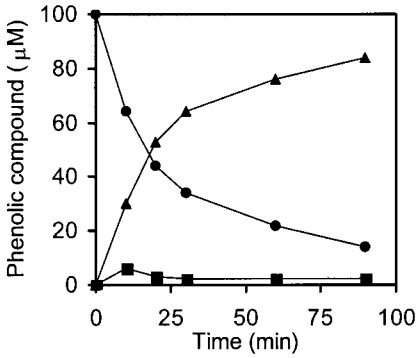

Figure 4. HPLC analysis of the VAO-mediated conversion of creosol. The reaction mixture contained 100 μM creosol and 1 μM VAO in 50 mM potassium phosphate buffer, pH 7.5, and was incubated at 25 °C. The concentrations of creosol (●), vanillyl alcohol (■), and vanillin (▲) were determined from reference solutions.

from other VAO-catalyzed oxidations (*13*). It should be noted here that the pH optimum of the reaction with creosol was determined at a substrate concentration of 150 μM. At higher substrate concentrations the overall conversion rate decreased due to the competitive binding of creosol, inhibiting the conversion of the intermediate product vanillyl alcohol.

Table 1 shows that the catalytic efficiency of VAO with creosol at pH 7.5 is 15-fold lower compared to the efficiency with vanillyl alcohol. This suggests that the slow hydroxylation of creosol limits the synthesis of vanillin. Therefore, we studied the initial enzymatic conversion of creosol to vanillyl alcohol in more detail. This reaction can be described by two half-reactions. First, the enzyme-bound flavin cofactor is reduced by the substrate, forming a complex between the reduced enzyme and the *p*-quinone methide intermediate of creosol (eq 1). In the second step, the reduced enzyme is reoxidized by molecular oxygen and water attacks the intermediate product, forming vanillyl alcohol (eq 2).

$$E_{ox} + \text{creosol} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}\sim\text{creosol} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{red}\sim\text{quinone methide} \quad (1)$$

$$E_{red}\sim\text{quinone methide} + O_2 + H_2O \xrightarrow{k_3} E_{ox} + \text{vanillyl alcohol} + H_2O_2 \quad (2)$$

In most VAO-catalyzed reactions studied so far, the reductive half-reaction limits catalysis (*20*, *26*). When creosol and VAO were mixed in the stopped-flow spectrophotometer under anaerobic conditions, a monophasic decrease in absorbance at 439 nm was observed, indicative of the reduction of enzyme-bound flavin. The rate of reduction was dependent on the concentration of creosol with a dissociation constant $K_d = 159 \pm 34$ μM at pH 7.5 (Figure 3). At low substrate concentrations the reduction rate reached a finite value of 0.16 s^{-1}, suggesting that the reduction is a reversible process in which $k_{-2} = 0.16 \pm 0.01$ s^{-1} (*20*, *27*). The forward reduction rate (k_2) was calculated to be 0.30 ± 0.02 s^{-1}, which is 4-fold higher than the turnover rate of VAO with this substrate. This strongly indicates that the reductive half-reaction, in analogy to the VAO-mediated conversion of *p*-cresol, only partially limits catalysis (*20*).

When VAO was mixed with creosol under aerobic conditions, the redox state of the flavin cofactor could be followed spectrophotometrically at 439 nm. We found that the enzyme-bound flavin was mainly in the reduced state during turnover at pH 7.5 (66%), again indicating that the reductive half-reaction does not limit the turnover rate. Moreover, upon excitation at 360 nm of the aerobic complex between creosol and VAO, a strong fluorescence emission with a maximum at 470 nm was observed. In line with earlier results, this suggests the formation of an abortive covalent flavin−substrate adduct, which is stabilized under aerobic conditions (*20*).

As mentioned previously, the VAO-mediated turnover of creosol at pH 10 was 10-fold slower than that at pH 7.5 (Figure 2). When VAO and creosol were mixed at pH 10 under aerobic conditions, the flavin was almost completely in the reduced state (95%). Moreover, the fluorescence emission spectrum upon excitation at 360 nm became stronger at pH 10 compared to pH 7.5. These results clearly support the proposition that the decreased turnover rate of creosol at high pH values is caused by the increased stability of the abortive creosol−flavin adduct.

When the VAO-mediated conversion of creosol was followed by HPLC, only low concentrations of vanillyl alcohol were detected at pH 7.5 and 10 (Figure 4). This is in agreement with the slow conversion of creosol relative to vanillyl alcohol. At both pH 7.5 and 10 the final yield of vanillin was 100% when the reaction was performed at a starting substrate concentration of 100 μM, as determined by HPLC and spectral analysis.

Vanillylamine Production. Capsaicin, the pungent principle of red pepper, is a cheap feedstock for the production of vanillylamine. In agreement with earlier reports (*16*, *17*), we observed that capsaicin can be metabolized by enzymes from liver via vanillylamine to the end products vanillyl alcohol and vanillic acid. In our experiments capsaicin was converted by rat liver microsomes to vanillylamine at a rate of 1.2×10^{-3} mM/min/mg of protein. Potentially, the amide bond in capsaicin can also be hydrolyzed by other hydrolases. Therefore, several commercially available enzymes were tested for their capacities to hydrolyze capsaicin. Among these enzymes (see Materials and Methods) only penicillin G acylase was able to hydrolyze capsaicin at a rate of 3.5×10^{-6} mM/min/mg. Vanillylamine was detected as the single aromatic product. For comparison, when 5 mM capsaicin was incubated in 100 mM NaOH at 95 °C, the chemical hydrolysis proceeded with a pseudo-first-order rate of 2.0×10^{-3}/min, whereas in 10 mM NaOH at 95 °C no hydrolysis occurred.

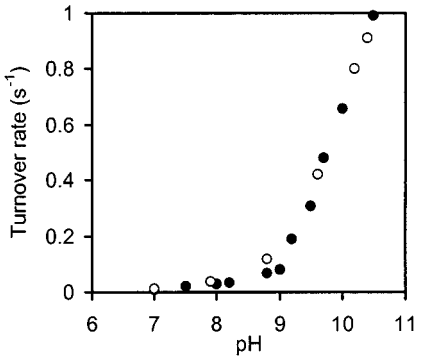

Figure 5. pH dependence of the reaction of VAO with vanillylamine. VAO activity was monitored either by spectral analysis of vanillin production (●) or oxygen consumption (○). The reactions were performed in 50 mM potassium phosphate buffer (pH 7.0−7.8), 50 mM Tris/H_2SO_4 (pH 7.8−8.9), and 50 mM glycine/NaOH (pH 9.0−10.5) at 25 °C using 1 mM vanillylamine and 1 μM VAO for spectral studies or 4 μM VAO for oxygen consumption experiments.

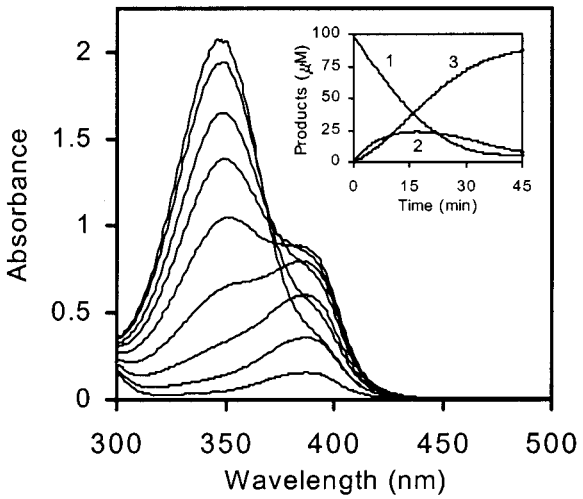

Figure 6. Enzymatic conversion of vanillylamine via vanillylimine to vanillin. The reaction mixture contained 50 mM glycine/NaOH, pH 9.0, 100 μM vanillylamine, and 0.5 μM VAO and was incubated at 25 °C. Spectra were taken at regular time intervals after the addition of VAO. The inset shows the estimated concentrations of (1) vanillamine, (2) vanillylimine, and (3) vanillin, respectively.

Conversion of Vanillylamine. In addition to creosol, vanillylamine obtained from capsaicin is a natural precursor of vanillin. The VAO-catalyzed conversion of vanillylamine involves the initial formation of vanillylimine. Subsequently, this intermediate product is hydrolyzed nonenzymatically to vanillin. When the conversion of vanillylamine by VAO was studied at pH 7.5, the turnover rate of the enzyme was very low (Table 1). This prompted us to study the pH dependence of the enzymatic reaction by oxygen consumption experiments as well as spectral analysis. With both methods we found similar pH-dependent turnover rates (Figure 5). The rate of the VAO-mediated conversion of vanillylamine increased dramatically when the pH of the reaction medium was raised to pH >9. Again, this pH profile is clearly distinct from that of other VAO-catalyzed reactions (*13*, *14*). Due to the protein instability it was not possible to measure reliable turnover rates at pH values >10.5. When VAO and vanillylamine were mixed aerobically, the redox state of the flavin cofactor could be followed spectrophotometrically. It was found that the enzyme is mainly in the oxidized state during turnover at both pH 7.5 (80%) and pH 9.7 (>90%), strongly indicating that, in contrast to the VAO-mediated conversion of creosol, the reductive half-reaction limits the turnover rate.

When the enzymatic deamination of vanillylamine was monitored spectrophotometrically, the initial formation of the intermediate product vanillylimine and its subsequent conversion to vanillin could be observed at 390 and 340 nm, respectively (Figure 6). The concentration of vanillylimine during catalysis was calculated using the molar absorption coefficient of vanillin (ϵ_{340} = 22.9 mM/cm at pH 9.0) and the absorbance changes in time due to vanillylimine and vanillin formation. The transient accumulation of relatively low amounts of vanillylimine is in line with the observation that the enzymatic formation of vanillylimine is the rate-limiting step in catalysis. For the VAO-mediated conversion of creosol, the yield of vanillin production reached almost 100%.

DISCUSSION

In the present study we have addressed the catalytic potential of VAO for the production of vanillin. A potentially attractive feedstock for the enzymatic formation of vanillin is creosol, which can be obtained from creosote (*18*, *19*). The VAO-mediated conversion of creosol to vanillin reaches a yield of 100%, but the conversion rate is rather low due to the formation of a nonreactive covalent adduct between creosol and the flavin prosthetic group of VAO. The covalent adduct slowly decomposes to vanillyl alcohol, thereby determining the overall turnover rate of VAO. The flavin−creosol adduct is more stable at basic pH values, hence shifting the pH optimum of VAO, which is normally around pH 10, to pH 7.5 (*13*, *26*). Moreover, the competitive binding of creosol inhibited the conversion of the intermediate product vanillyl alcohol to vanillin. As a consequence, the optimal conditions for the VAO-catalyzed conversion of creosol were found to be at pH 7.5 and a substrate concentration of 150 μM.

A second potentially attractive feedstock for the enzymatic production of vanillin is capsaicin. This pungent principle of red pepper can be easily obtained at low cost and can be hydrolyzed enzymatically to vanillylamine by a carboxylesterase from liver. This esterase has been found in the liver of chicken, hog, cow, and rat (*16*), and the rat liver enzyme has been purified and characterized to some extent (*17*). Capsaicin is not hydrolyzed by trypsin, peptidase, or aminoacylase (*16*). We found that capsaicin is extremely slowly hydrolyzed by penicillin G acylase from *E. coli*. The rate of this enzymatic hydrolysis reaction might be increased by performing the reaction in organic solvents, as the solubility of capsaicin would be enhanced.

Vanillylamine reacted slowly with VAO at neutral pH values but was efficiently converted between pH 9 and 10.5 with a high vanillin yield. This strong increase in VAO activity around pH 9 is unusual. As the rate-limiting step in the conversion of vanillylamine to vanillin is associated with the formation of the vanillylimine intermediate, the pH dependence of this reaction might be related to the binding of vanillylamine. For other VAO substrates, it is thought that the initial formation of the quinone methide intermediate (eq 1) is facilitated by the preferential binding of the phenolate form of the substrate (*28*). For example, when the substrate analogue isoeugenol binds to VAO, the pK_a

of the phenol decreases from 9.8 to 5.0 (*14*). Possibly, binding of vanillylamine to the enzyme does not stimulate phenol deprotonation. Alternatively, the pH effect might be related to the preferred binding of the phenolate form of vanillylamine. On the other hand, the protonation state of the amide moiety of vanillylamine might also be of importance for its reactivity.

In conclusion, this paper presents two novel enzymatic routes for the biocatalytic production of natural vanillin. The bienzymatic process from capsaicin to vanillin is most promising from a biotechnological point of view, as capsaicin is a widely available compound. To make this process more feasible, the first enzyme in the reaction sequence, a carboxylesterase, needs to be characterized in detail and overexpressed in a suitable host. The VAO-catalyzed deamination of vanillylamine is efficient, does not need any external cofactors, and uses molecular oxygen as a clean and mild oxidant. Moreover, the enzyme can be easily obtained in large amounts (*25*). When the two enzymes can be combined in a single reaction mixture, one can design a one-pot reactor to produce natural vanillin. Because the pH optima of these two enzymatic reactions vary between pH 8−9 for the carboxylesterase (*17*) and pH 10−10.5 for VAO, one can assume that the optimum pH conditions for a one-pot system are between pH 9 and 10.

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